UNIVERSITI PUTRA MALAYSIA

IMMUNOCHEMISTRY AND MOLECULAR APPROACHES TOWARDS IDENTIFICATION OF MALAYSIAN CYPRINID HERPESVIRUS

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By

SAMSON SOON MIN NGEN

Thesis Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy in the Faculty of Veterinary Medicine Universiti Putra Malaysia

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Abstract of thesis submitted to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

IMMUNOCHEMISTRY AND MOLECULAR APPROACHES TOWARDS IDENTIFICATION OF MALAYSIAN CYPRINID HERPESVIRUS

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Chairman: **Dr. Hassan Hj. Mohd. Daud, Ph.D**

Faculty : **Veterinary Medicine**

Immunochemistry and molecular approaches were used to identify a Malaysian cyprinid herpesvirus responsible for papilloma among Koi carps (*Cyprinus carpio* L.) and goldfish (*Carassius auratus* L.) in Malaysia. Immunochemistry approaches employing hybridoma technology established a hybridoma clone (DG3-1) producing specific IgM κ light chain monoclonal antibody (MAb) against Malaysian cyrprinid herpesvirus. The MAb was cross-reactive against Japanese cyprinid herpesvirus type 1 (CHV) antigens but not against Channel catfish herpesvirus (CCV) and Salmonid herpesvirus (SHV-2) in immunodot-blot assay. The cyprinid herpesvirus type-specific epitope recognised by the MAb was located on two viral polypeptides having the molecular weight of 58,000 and 67,000 daltons in Malaysian cyprinid herpesvirus and CHV through Western blot analysis. As the MAb showed no neutralization activity against virus infection in cell culture and glycosylation inhibitors did not affect the presence and migration of the antigens under polyacrylamide gel electrophoresis, evidences as such suggest the antigens are nonglycosylated components of the viral structure.

Immunohistochemical analysis on goldfish papilloma tissue sections with MAb using labeled avidin binding (LAB) method demonstrated specific staining of cyprinid herpesvirus antigens within the nucleus of infected cells. Specific localization of these viral antigens in the cell nuclei were consistent with reports of nonglycosylated herpesvirus antigens involving viral capsid components or DNA-binding proteins. Employing molecular techniques, cyprinid herpesvirus nucleic acid sequences were later confirmed to be present in the immunohistochemical positive papilloma sections through *in situ* hybridization assay using a 1,161 bp CHV nucleic acid probe.

Molecular identification by polymerase chain reaction (PCR) using CHV specific primers was extremely sensitive, specific, rapid and practical. The technique successfully amplified a 433 bp DNA fragment from frozen archival goldfish papilloma tissues and recent papillomas obtained from goldfish and carp hybrids. Nucleic acid sequencing of the DNA fragment revealed identical sequence homology with CHV, thus confirming conclusively that Malaysian cyprinid herpesvirus and CHV are members of the same group of virus. Detection sensitivity level as assessed with first step PCR, was capable of detecting viral nucleic acids from 1 fg or 200 copies of actual viral target sequences and from as low as 1-10 virus infected cells. Sensitivity level was increased 100-1000-fold when nested PCR strategy was employed. Specificity of detection evaluated by DNA fragment polymorphism demonstrated homologous DNA sequences among cyprinid herpesvirus representatives from Malaysia, Israel and Japan. A quantitative competitive PCR assay based on the current viral target sequence also provided quantitative description of infection and viral burden with preliminary results indicative of CHV possessing an alphaherpesvirus gene-like expression kinetics.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan bagi mendapatkan Ijazah Doktor Falsafah

PENDEKATAN IMUNOKIMIA DAN MOLEKUL DALAM PENGENALPASTIAN HERPESVIRUS CYPRINID MALAYSIA

Oleh

SAMSON SOON MIN NGEN

Mei 2001

Pengerusi: **Dr. Hassan Hj. Md. Daud, Ph.D**

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Kaedah imunokimia dan molekul telah digunakan dalam pengenalpastian herpesvirus cyprinid Malaysia, yang bertanggungjawab ke atas kejadian papiloma di dalam ikan koi (*Cyprinus carpio* L.) dan ikan emas (*Carassius auratus* L.) di Malaysia. Kaedah imunologi menggunakan teknologi hibridoma telah menghasilkan klon hibridoma (DG3-1) yang mengeluarkan antibodi monoklon (MAb) IgM dengan rantai κ yang spesifik terhadap cyprinid herpesvirus Malaysia. Antibodi monoklon menunjukkan reaksi saling terhadap antigen cyprinid herpesvirus type 1 (CHV) Jepun tetapi tidak terhadap Channel catfish herpesvirus (CCV) and Salmonid herpesvirus (SHV-2) dalam asei "immunodot-blot". Analisis "Western blot" mendedahkan bahawa epitope spesifik cyprinid herpesvirus yang dikenalpasti oleh MAb terletak pada dua polipeptida virus dengan berat molekul 58,000 and 67,000 dalton pada cyprinid herpesvirus Malaysia dan CHV. Oleh kerana MAb tidak menunjukkan neutralisasi terhadap jangkitan virus di dalam kultur tisu dan rawatan penyekat glikosilasi tidak mempengaruhi kehadiran dan migrasi antigen-antigen dalam elektrophoresis gel polyacrylamide, menunjukkan bahawa

antigen-antigen ini adalah komponen struktur teras virus yang terdiri dari polipeptida tidak berglikosilasi.

Analisis imunohistokimia ke atas keratan tisu papiloma ikan emas menggunakan teknik "Labeled Avidin Binding" (LAB) menunjukkan pewarnaan spesifik antigen cyprinid herpesvirus di dalam nukleus sel yang dijangkiti. Pengesanan antigen-antigen ini di dalam nukleus sel adalah selari dengan laporan mengenai antigen tidak berglikosilasi herpesvirus yang terdapat pada komponen kapsid virus dan protin pengikat DNA. Pendekatan teknik molekul terhadap keratan immunohistokimia papiloma yang positif menggunakan prob asid nukleik CHV bersaiz 1,161 bp dengan kaedah hibridisasi "*in situ*" turut menunjukkan kehadiran asid nukleik CHV.

Pengenalpastian melalui reaksi polimeras berantai (PCR) dengan primer spesifik CHV juga didapati sangat sensitive, spesifik, cepat dan praktikal. Kaedah ini berjaya menghasilkan fragmen DNA bersaiz 433 bp dari tisu papiloma yang dibekukan dan yang baru dari ikan emas dan kap hibrid. Penjujukan asid nukleik menunjukkan homologi yang sama dengan CHV justeru mengesahkan bahawa MCHV dan CHV adalah virus yang sama. Sensitiviti pengesanan dengan PCR dengan teknik PCR tahap satu mampu mengesan asid nukleik CHV dari 1 fg atau 200 salinan sasaran jujukan asal virus dan dari 1-10 sel terjangkit. Sensitiviti pengesanan ini dapat dipertingkatkan 100-1000 kali ganda dengan kaedah PCR bersarang. Spesifisiti pengesanan PCR dengan kajian pecahan polimofisa DNA menunjukkan jujukan asid nukleik serupa di antara cyprinid herpesvirus dari Malaysia, Israel dan Jepun. Kaedah kuantitatif PCR berdasarkan sasaran jujukan virus PCR yang digunakan membolehkan gambaran kuantitatif terhadap tahap

jangkitan dan beban virus diselidiki, di mana keputusan awal menunjukkan bahawa CHV memiliki expresi kinetik gen yang seakan sama dengan kumpulan alphaherpesvirus.

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I certify that an Examination Committee met on 2nd May 2001 to conduct the final examination of Samson Soon Min Ngen on his Doctor of Philosophy thesis entitled "Immunochemistry and Molecular Approaches Towards Identification of Malaysian Cyprinid Herpesvirus" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy.

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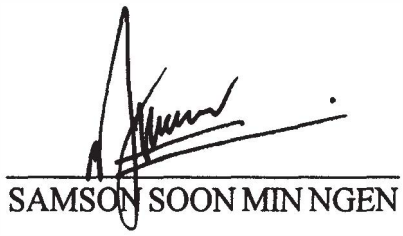
Date: 12 JUL 2001

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



SAMSON SOON MIN NGEN

Date: 21st June 2001

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ABC	-	Avidin-biotin complex
ABTS	-	2,2'-Azino-di-(3-ethyl-benzthiazoline-6)
ag	-	Attogram
BB	-	Brown Bullhead
BCIP	-	Bromochloroindolyl phosphate
bp	-	Base pairs
BSA	-	Bovine serum albumin
CCV	-	Channel Catfish Virus
CHV	-	Cyprinid Herpesvirus
cm^2	-	Centimeters square
CPE	-	Cytopathic Effect
cDNA	-	Complementary Deoxyribonucleic Acid
CPCR	-	Competitive Polymerase Chain Reaction
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic Acid
ELISA	-	Enzyme-linked immunosorbent assay
EPC	-	*Epithelioma Papulosum Cyprini*
EHV-1	-	Equine Herpesvirus Type One
EHV-2	-	Equine Herpesvirus Type Two
EHV-4	-	Equine Herpesvirus Type Four